*Immunology* 1985 **54** 449

# Inhibition of C3 deposition on solid-phase bound immune complexes by lactoferrin

F. KIEVITS & A. KIJLSTRA *Department of Ophthalmo-Immunology, Netherlands Ophthalmic Research Institute, Amsterdam, The Netherlands*

*Accepted for publication 29 October 1984*

**Summary.** In the study described here, the effect of human tears and purified lactoferrin was investigated on the deposition of complement components on solid-phase bound immune complexes.

After incubating immune complexes with fresh normal human serum, the deposition of complement components (C3, C4 and C5) was measured with an ELISA technique. Rabbit antibodies were used as a constituent of the immune complexes, and so both alternative and classical complement pathway activation could be studied. The addition of human tears or purified lactoferrin to this system resulted in the inhibition of classical pathway deposition of C3 and C5, while C4 deposition was not affected.

High concentrations of human tears also inhibited alternative pathway C3 deposition on immune complexes, whereas lactoferrin did not detectably affect this pathway. The inhibition of complement activation by tears was not due to a masking of the immune complexes or the already deposited C3. Experiments with purified lactoferrin furthermore showed that lactoferrin did not bind to the complexes, either before or during complement activation. These findings suggest that the complement inhibitory effect is probably taking place in the fluid phase. Saturation of lactoferrin with iron or copper ions resulted in a markedly diminished effect on the capacity of lactoferrin to inhibit complement activation.

C4 deposition on immune complexes was not affected by lactoferrin, which suggested that the inhibition of the classical pathway was due to an effect on the classical C3 convertase. The fact that lactoferrin inhibits the classical, but not the alternative C3 convertase, suggests that the effect is probably not mediated through a competition for certain trace metal ions, but may be caused by protein-protein interactions.

The findings reported here indicate that lactoferrin may play an important anti-inflammatory role by modulating activation of the complement system. This observation adds a new property to the already described functions of the iron-binding protein lactoferrin.

Correspondence: Dr A. Kijlstra, Dept. Ophth. Immunology, Neth. Ophth. Res. Institute, P.O. Box 12141, 1100 AC Amsterdam, The Netherlands.

## INTRODUCTION

Human tears contain a variety of proteins involved in the protection of the eye against invading microorganisms. The proteins lactoferrin and lysozyme are responsible for a non-immunological, whereas secretory IgA mediates a specific immunological defence mechanism in the tear film (van Haeringen, 1981). The role of the complement system as an effector mechanism of humoral immunity in the tear film is not yet clear. Tears obtained from healthy eyes were not shown to contain detectable classical haemolytic complement activity (Kijlstra & Veerhuis, 1981), while tears obtained from inflamed eyes showed a marked increase in haemolytic activity of individual components C1, C4, C3 and C5 (Mondino & Zaidman,

1983). Whether tears obtained from inflamed eyes contain total haemolytic complement activity is not yet known.

Instead of containing total haemolytic complement activity, normal tears obtained from humans (Veerhuis & Kijlstra, 1982) and various other species (Kijlstra, Jeurissen & Koning, 1984) were shown to contain powerful inhibitors of the classical complement pathway. The major inhibiting effect in human tears was associated with the iron-binding protein lactoferrin (Veerhuis & Kijlstra, 1982). Further studies showed that the inhibition of haemolytic complement activation by lactoferrin was due to the blocking of the assembly of the classical C3 convertase (Kijlstra & Jeurissen, 1982), thus preventing the cleavage of C3.

In the present investigation, the effect of lactoferrin on the deposition of C3 and C4 on solid-phase bound immune complexes was examined during incubation with fresh human serum. The results show that lactoferrin inhibits classical, but not alternative, complement pathway deposition of C3. Furthermore, it was shown that saturation of lactoferrin with certain metal ions can abolish the anti-complementary activity.

## MATERIALS AND METHODS

*Tears*

Tears were collected with 50 $\mu$l micropipettes from staff members of our institute after stimulating the secretion with a stream of pressurized air. A pool of these tear samples was stored at $-20°$.

*Lactoferrin*

Lactoferrin was isolated from human milk by chromatography on Heparin-Sepharose (Pharmacia, Uppsala, Sweden) (Boesman & Finkelstein, 1982). Its purity and molecular weight were verified by SDS polyacrylamide gel (7·0%) electrophoresis using standard calibration proteins (Bio-Rad, Richmond CA). The percentage of iron saturation, estimated by titration with iron-nitrilotriacetic acid and measurement of absorbance at 465 nm was near 10% (Gordon, Ziegler & Basch, 1962; Bates, Billups & Saltman, 1967). In most experiments, lactoferrin was used as such.

In order to study the effect of saturation of lactoferrin with various metal ions, the following procedure was carried out. Lactoferrin (4 mg/ml) was incubated (60 min, 4°) with 50 $\mu$l of metal chelates in phosphate-buffered saline (PBS) at pH 7·4. Non-bound metal ions were removed by dialysis overnight against PBS at 4°. Fe(III)-chelates were prepared by dissolving 0·4 g $Fe(NO_3)_3.9H_2O$ in 100 ml distilled water containing 0·2 g nitrilotriacetic acid (NTA; ICN Pharmaceuticals, Plainview, NY) and adjusting the pH to 7·4 by adding solid $NaHCO_3$ (Bates *et al.*, 1967). The other metal chelates were prepared by dissolving 0·3 g $ZnSO_4.7H_2O$, 0·22 g $MnSO_4.4H_2O$ or 0·5 g $CuSO_4.5H_2O$ in 100 ml distilled water containing 0·2 g citric acid (Masson & Heremans, 1968; Ainscough *et al.*, 1980).

Protein concentration was measured by the Lowry method using crystalline bovine serum albumin as a standard.

Lactoferrin concentrations were determined quantitatively by an ELISA method as described earlier (Kijlstra, Jeurissen & Koning, 1983).

*Complement deposition on immune complexes*

Polystyrene microcuvettes (Gilford, Cleveland, OH) were coated with 300 $\mu$l human serum albumin (HSA; Central Laboratory of the Red Cross Bloodtransfusion Service, Amsterdam, The Netherlands) diluted in coating buffer (1·6 g $Na_2CO_3$, 2·9 g $NaHCO_3$, 0·2 g $NaN_3$ in 1 liter $H_2O$, pH 9·6) at a final concentration of 75 $\mu$g/ml. The microcuvettes were incubated at 37° in a shaking water bath for 1 hr and washed three times with posphate-buffered saline (PBS) containing 0·1% Tween 20 (PBS-Tween). Then 300 $\mu$l of a 1:50 dilution (in PBS-Tween) of inactivated (30 min, 56°) rabbit anti-HSA antiserum were added. After an incubation at 37° for 1 hr and three washes, the cuvettes were inverted on a dry paper towel and allowed to drain thoroughly. The immune complex (Icx) coated cuvettes were used immediately or stored dry at 4° and used within 5 days.

Activation of the classical complement pathway took place by incubation (60 min, 37°) of Icx coated cuvettes with 0·5% of fresh normal human serum (NHS, 300 $\mu$l) diluted in half isotonic veronal-buffered saline containing 2·5% dextrose, 0·1% gelatin, 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$ ($DGVB^{++}$).

Activation of the alternative pathway took place by incubation of Icx with 2% NHS diluted in $DGVB^{++}$, containing 8 mM EGTA. After incubation at 37° for 1 hr and three washes with PBS-Tween, C3 deposition was developed with 300 $\mu$l peroxidase-coupled goat anti-human C3c IgG (1:500, Nordic, Tilburg, The Netherlands) diluted in PBS-Tween and incubated for 60 min at 37°.

C4 and C5 deposition were investigated after incubation (60 min, 37°) with 300 μl goat anti-human C4 (1:3000, Nordic) or C5 antiserum (1:250, Nordic) followed by an incubation (60 min, 37°) with 300 μl peroxidase conjugated rabbit anti-goat IgG (1:10,000, Nordic). After washing three times with PBS-Tween, bound peroxidase labelled antibody was developed at room temperature by adding 350 μl ABTS solution (0·16 mM 2·2-azino-di-3-ethyl-benzthiazoline-6-sulphonate, Boehringer, Mannheim, W. Germany; 0·12% $H_2O_2$ in 0·05 M citric acid, pH 4·0). The reaction was stopped by adding 50 μl 10% SDS to each cuvette.

The green reaction product was measured in a spectrophotometer (EIA, Gilford, Cleveland, OH) at 405 nm.

The effect of normal human tears or purified human lactoferrin on C3, C4 and C5 deposition on solid phase bound Icx was tested by incubating (60 min, 37°) 150 μl of tears or lactoferrin dilutions with 150 μl of diluted NHS in the Icx coated microcuvettes.

Controls included uncoated cuvettes, HSA coated cuvettes and Icx coated cuvettes, which were incubated with $DGVB^{++}$ alone, inactivated NHS (30 min, 56°) and fresh NHS.

All samples were analysed in duplicate and corrected for the $DGVB^{++}$ control, which ranged between 0·250 and 0·300 OD units.

## RESULTS

### C3 and C4 deposition on solid phase bound immune complexes

Complement activation by solid-phase bound immune complexes was investigated by measuring binding of complement components to these complexes with an ELISA-technique. Deposition of C3 and C4 was measured after incubating immune complexes with various concentrations of fresh normal human serum (300 μl, 0·25–2%, 60 min, 37°). Figure 1 shows a dose-dependent C3 and C4 deposition after incubation with fresh human serum, whereas no deposition was seen after incubation with heat-inactivated (30 min, 56°) human serum.

The deposition of C5 followed a similar but lower profile as C4 (data not shown). Deposition of C3 after activation of the alternative pathway was investigated using buffers in the presence of 8 mM EGTA. Using the EGTA buffer, deposition of C3 on immune complexes was detectable at final serum concentrations of 1% or more (Fig. 2). Under these conditions, no C4 deposition was detectable, indicating a selective activation of the alternative pathway.

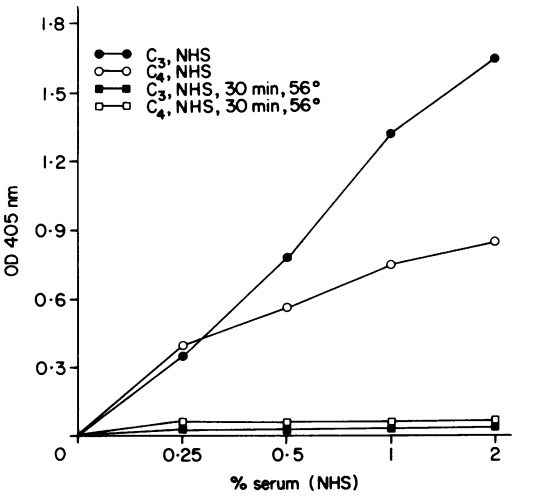


**Figure 1.** Deposition of C3 and C4 on solid-phase bound immune complexes after incubation with fresh (NHS) or heated (NHS, 30 min, 56°) normal human serum.

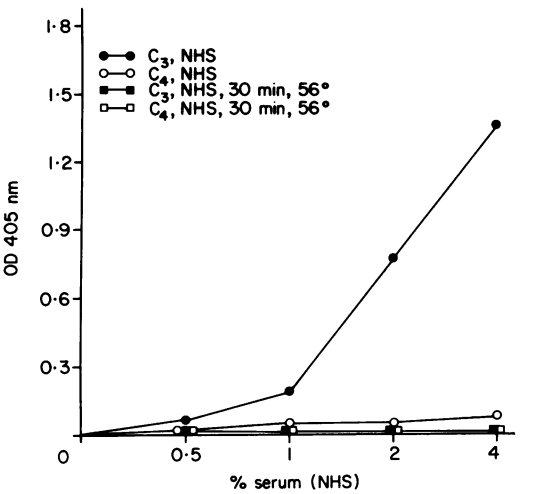


**Figure 2.** Deposition of C3 and C4 on solid-phase bound immune complexes after incubation with fresh (NHS) or heated (NHS, 30 min, 56°) normal human serum in the presence of 8 mM EGTA.

### C3 deposition in the presence of human tears

Previous studies have demonstrated that tears contain factor(s) that can inhibit classical haemolytical complement activity (Kijlstra & Veerhuis, 1981). In order

to investigate the effect of human tears on complement activation by solid-phase bound immune complexes, the following experiments were performed, using the ELISA-technique as described above.

Immune complex-coated microcuvettes were incubated (60 min, 37°) with 150 μl of human serum (final concentrations 0·5% or 2%) in the presence of 150 μl of various dilutions of human tears (final concentrations 0–50%), diluted in $DGVB^{++}$ or $DGVB^{++}$ containing 8 mM EGTA.

The effect of tears on classical pathway activation is represented by the experiment performed in the presence of 0·5% serum, whereas the effect on the alternative pathway was investigated with a serum concentration of 2% in EGTA buffer. The effect of tears on both pathways was studied using a serum concentration of 2% in a $DGVB^{++}$ buffer without EGTA.

A marked inhibition (57%) of classical pathway C3 deposition was seen at a concentration of 10% tears, whereas 40% tears had a similar inhibitory effect (60%) on alternative pathway C3 deposition (Fig. 3). Maximal inhibition of classical pathway C3 deposition occurred at tear concentrations between 20% and 30%, while higher concentrations resulted in a lower inhibition rate. A similar, although lower, maximal inhibitory effect with a subsequent decline was seen when the effect of tears on combined classical and alternative pathway C3 deposition was studied. In a separate similar experiment as described above, it was shown that 10% tears also caused an inhibition (41%) of the classical C5 deposition.

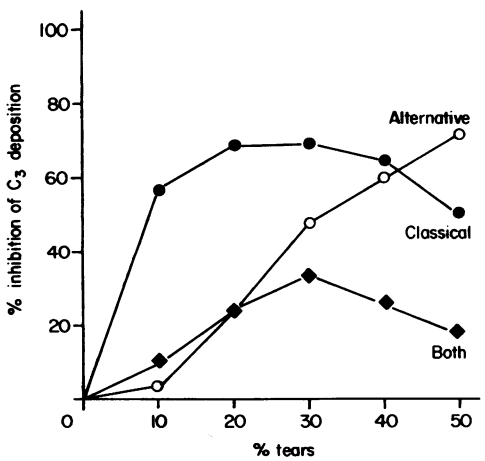


**Figure 3.** Inhibition of C3 deposition on solid-phase bound immune complexes after incubation with 0·5% human serum (classical pathway), 2% human serum with 8 mM EGTA (alternative pathway) or 2% human serum (both pathways) in the presence of various amounts of tears.

The inhibition of C3 deposition on solid phase bound immune complexes could be caused by:

(i) a direct effect of tears on complement activation;

(ii) an effect of tears on solid-phase bound immune complexes;

(iii) a masking effect of tears on immune complex bound C3, resulting in an inhibitory binding of the anti-C3 antibodies used in the ELISA.

The latter two possibilities were examined in the following experiment.

Solid-phase bound immune complexes were preincubated (60 min, 37°) with human tears (10%, 300 μl) or buffer ($DGVB^{++}$, 300 μl) and after washing (three times with PBS-Tween), a same incubation period was followed with fresh normal human serum (0·5%, 300 μl).

The results presented in Table 1 show that preincubation with tears had no influence on the classical C3 deposition. Preincubation of the immune complexes with serum (0·5%, 300 μl) followed by an incubation with tears (10%, 300 μl) also showed no inhibitory effect on the C3 deposition.

Controls included incubations with serum and tears together, heat-inactivated serum, or buffer alone (Table 1).

The observation that human tears did not mask the immune complexes or the already bound C3 indicated that the inhibition of C3 deposition by human tears was a direct effect of tears on complement activation.

### Effect of lactoferrin on the C3 and C4 deposition on immune complexes

Earlier studies using haemolytic assays showed that lactoferrin was the main complement inhibitor in human tears. Sucrose gradient ultracentrifugation of human tears showed that the peak which inhibited C3 deposition on immune complexes (as detected by the ELISA method) coincided with the lactoferrin peak (data not shown). The effect of purified human lactoferrin on alternative pathway, classical pathway and combined pathway C3 deposition, and C4 deposition on solid phase bound immune complexes, was investigated in a similar experiment as shown in Fig. 3. Immune complexes were incubated (60 min, 37°) with 150 μl of human serum (final concentration 0·5% or 2%) in the presence of 150 μl of various concentrations

**Table 1.** Is the inhibition of C3 deposition on immune complexes caused by masking the complexes or the already deposited C3 by tears?

| Experimental procedure* | | | C3 deposition† |
|---|---|---|---|
| Icx + buffer | wash | NHS | 1·113 |
| Icx + tears | „ | NHS | 1·047 |
| Icx + buffer | „ | NHS, 30 min, 56° | 0·327 |
| Icx + buffer | „ | buffer | 0·284 |
| Icx + buffer | „ | NHS + tears | 0·536 |
| Icx + NHS | „ | buffer | 1·052 |
| Icx + NHS | „ | tears | 1·019 |
| Icx + NHS, 30 min, 56° | „ | buffer | 0·329 |
| Icx + NHS + tears | „ | buffer | 0·599 |

* Solid-phase bound immune complexes (Icx) were preincubated (60 min, 37°) with buffer (DGVB$^{++}$), human tears (10%), fresh (NHS) or heated (NHS, 30 min, 56°) normal human serum (0·5%) and with NHS and tears together. After washing three times, a same incubation period was followed with fresh or heated NHS (0·5%), human tears (10%), NHS and tears together, or with buffer alone.

† C3 deposition was measured in a spectrophotometer at 405 nm.

of purified human lactoferrin (0·25–2 mg/ml) in the presence or absence of 8 mM EGTA.

Figure 4 shows that lactoferrin markedly inhibited classical C3 deposition, but had no detectable effect on C4 deposition on the complexes. Lactoferrin was also shown to inhibit classical C5 deposition (data not shown). At the concentrations of lactoferrin used, no detectable effect was seen on alternative pathway C3 deposition.

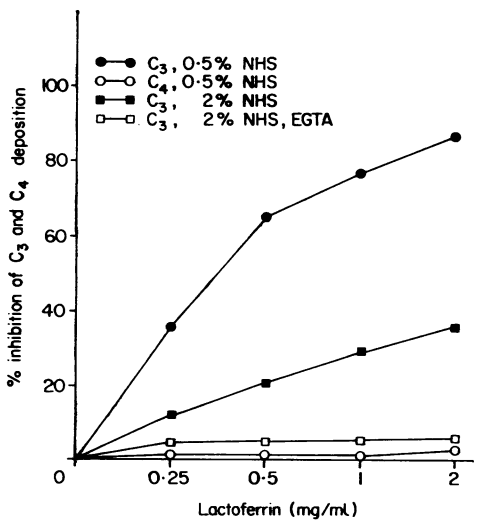


**Figure 4.** The effect of purified lactoferrin on the C3 and C4 deposition on solid-phase bound immune complexes after incubation with 0·5% normal human serum (NHS, classical pathway), 2% NHS with 8 mM EGTA (alternative pathway) or 2% NHS (both pathways).

In order to investigate whether a possible binding of lactoferrin to the immune complexes occurred before or after complement activation, the following experiment was performed. Solid-phase coated immune complexes were incubated with lactoferrin alone, serum alone or a combination of lactoferrin and serum, whereafter the deposition of C3, C4 and lactoferrin was determined. As a positive control, lactoferrin-coated microcuvettes were also included in the experiment. Table 2 shows that no lactoferrin was bound to any of the experimental microcuvettes except the positive control. Deposition of C3 was inhibited in the presence of lactoferrin, whereas no inhibition of C4 was observed, which is in agreement with the data shown in Fig. 4. These experiments suggested that lactoferrin probably exerts its complement inhibitory effect in the fluid phase.

### Inhibition by metal-substituted lactoferrin

The experiments shown above were performed with native human lactoferrin which was only 10% saturated with metal ions.

In order to investigate the effect of metal-saturated lactoferrin on its complement inhibitory effect, lactoferrin was preincubated with various metal ions, such as $Fe^{3+}$, $Cu^{2+}$, $Zn^{2+}$ and $Mn^{2+}$, whereafter unbound

metal ions were removed by dialysis. These various forms of saturated lactoferrin and of unsaturated native lactoferrin were incubated with solid phase bound immune complexes in the presence of 0·5% fresh serum, whereafter deposition of C3 was quantitated. The results (Fig. 5) show that the inhibition of classical pathway C3 deposition by native lacroferrin is markedly diminished when lactoferrin was saturated with $Fe^{3+}$ or $Cu^{2+}$ ions, whereas no substantial effects were seen by preincubation of lactoferrin with $Zn^{2+}$ or $Mn^{2+}$ ions.

**Table 2.** Does lactoferrin bind to solid-phase bound immune complexes?

| Experimental procedure* | | Deposition (OD 405 nm)† | | |
|---|---|---|---|---|
| Microcuvettes coated with: | Incubation with: | C3 | C4 | LF |
| Icx | NHS | 1·119 | 0·747 | 0·000 |
| Icx | NHS+LF | 0·590 | 0·783 | 0·001 |
| Icx | LF | 0·036 | 0·036 | 0·005 |
| LF | buffer | ND‡ | ND | 0·763 |

* Microcuvettes were coated with immune complexes (Icx) or with lactoferrin (LF, 5 μg/ml). The Icx coated cuvettes were incubated with normal human serum (NHS, 1%), lactoferrin (1 mg/ml) or with NHS and lactoferrin together. The lactoferrin coated cuvettes were incubated with buffer ($DGVB^{++}$) alone.

† All values are corrected for the control (Icx + $DGVB^{++}$).

‡ ND, not done.

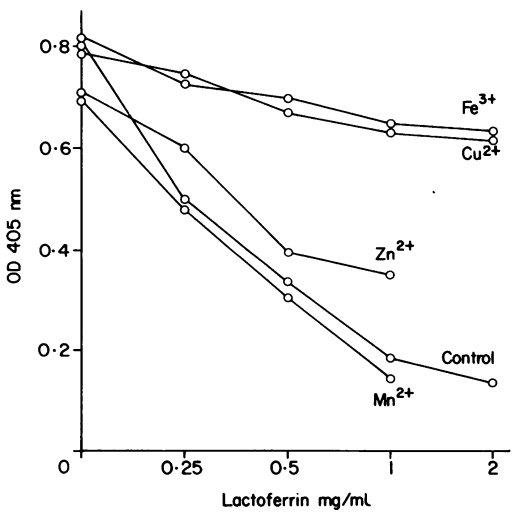


**Figure 5.** The effect of metal saturated lactoferrin on its capacity to inhibit C3 deposition on solid-phase bound immune complexes.

## DISCUSSION

The studies described here show that lactoferrin and human tears can inhibit the classical C3 deposition on solid-phase bound immune complexes, whereas the alternative pathway is only inhibited by high concentrations of tears and not by lactoferrin.

Preincubation of the immune complexes with serum or tears showed that inhibition of C3 deposition by tears is caused by a direct effect of tears on complement activation, and not by masking the immune complexes or the already deposited C3. Earlier investigations (Veerhuis & Kijlstra, 1982; Kijlstra & Jeurissen, 1982) and sucrose gradient ultracentrifugation experiments performed during the study described here have shown that the main complement inhibition in human tears was associated with lactoferrin. These findings suggest that lactoferrin, which is present in human tears (Kijlstra, Jeurissen & Koning, 1983), and many other external secretions (Masson, Heremans & Dive, 1966) but also in the granules of polymorphonuclear neutrophils (Masson, Heremans & Schonne, 1969) may be a specific inhibitor of the classical complement pathway. Besides the already known antibacterial properties (Bullen, Rogers & Leigh, 1972; Arnold *et al.*, 1982), lactoferrin may therefore also have an anti-inflammatory function by modulating the activation of the complement system.

Our experiments demonstrate that the anti-complementary effect of lactoferrin is not due to an inhibition of C1 activation, because C4 deposition on the immune complexes was not affected in the presence of lactoferrin. The fact that the classical pathway C3 deposition was inhibited, whereas C4 deposition was not altered, indicates a blockade of the assembly of the classical C3 convertase or an inhibitory effect on the C3 activation by this convertase. These observations are in agreement with our previous studies (Kijlstra & Jeurissen, 1982) using haemolytic assays, whereby lactoferrin was shown to inhibit the formation of the EAC142 intermediate. These findings suggest that lactoferrin will also prevent the formation of the biologically active mediators of complement activation such as C3a and C5a.

The fact that, in our study, no binding of lactoferrin

was seen to solid phase complexes which had been incubated in the presence of fresh serum, indicates that the inhibiting activity of lactoferrin does not take place on the activator surface but most likely in the fluid phase. In the fluid phase, lactoferrin could directly interact with complement factors or with metal ions which are essential for the assembly of the classical C3 covertase. The anti-complementary effect of lactoferrin was shown to be dependent on the saturation level of the protein with iron or copper ions, which suggests that the observed effect could be due to a depletion of metal ions from the medium. The fact that lactoferrin can inhibit the classical, but not the alternative, C3 convertase would argue against a competitive effect between lactoferrin and the C3 convertase for certain metal ions. If this were the case, lactoferrin would also be expected to inhibit the alternative pathway C3 convertase. Lactoferrin is known to possess remarkable protein binding properties (Hekman, 1971), and it is therefore possible that lactoferrin may bind to complement factors in the fluid phase. Further experiments are, however, necessary to clarify this issue.

The observation that the anti-complementary effect of lactoferrin is abolished when the protein is satured with metal ions suggests two possible mechanisms of action. The first mechanism, which was already mentioned above, would suggest a competitive effect for certain metal ions. The second mechanism would involve structural changes after the uptake of metal ions, leading to functional changes. Others have shown that saturation of lactoferrin with metal ions results in small local conformational changes (Mazurier *et al.*, 1976). These local conformational changes could possibly affect the regulation of complement activation by lactoferrin.

Of interest is the observation that lactoferrin will only interact with its receptor on macrophages and monocytes unless it has been iron-saturated (Broxmeyer *et al.*, 1981; Bennet & Davis, 1981; van Snick & Mason, 1976), also suggesting a role of iron in the three-dimensional structure of the lactoferrin molecule.

The involvement of iron-binding sites in the anti-complementary effect of lactoferrin is not yet clear, but experiments with anti-lactoferrin monoclonal antibodies may give more insight in this matter in the near future.

The inhibition of C3 deposition on solid phase bound immune complexes, as described in this report, adds another new property to the list of known effects of the iron-binding protein lactoferrin.

## ACKNOWLEDGMENTS

We thank Mrs J. C. M. Beentjes, Mrs E. Wilmink and Mr I. Erdös for their assistance during the preparation of the manuscript.

## REFERENCES

AINSCOUGH E.W., BRODIE A.M., PLOWMAN J.E., BOOR S.J., LOEHR J.S. & LOEHR T.M. (1980) Studies on human lactoferrin by electron paramagnetic resonance, fluorescence and resonance raman spectroscopy. *Biochemistry*, **19**, 4072.

ARNOLD R.R., RUSSEL J.E., BREWER W.J. & GAUTHIER J.J. (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. *Infect. Immun.* **35**, 792.

BATES G.W., BILLUPS C. & SALTMAN P. (1967) The kinetics and mechanism or iron (III) exchange between chelates and transferrin. *J. biol. Chem.* **12**, 2810.

BENNET R.M. & DAVIS J. (1981) Lactoferrin binding to human peripheral blood cells: an interaction with a B-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. *J. Immunol.* **127**, 1211.

BOESMAN M. & FINKELSTEIN R.A. (1982) Sequential purification of lactoferrin, lysozyme and secretory immunoglobulin A from human milk. *FEBS* Lett. **144**, 1.

BROXMEYER H.E., PELUS L.M., GILLUS S., KINCADE P., DESOUSA M. & MOORE M.A.S. (1981) Heterogeneity in responsiveness of mononuclear phagocytes to receptor-binding of lactoferrin and to inhibition of the production of granulocyte-macrophage colony stimulating factors by lactoferrin. In: *Heterogeneity of Mononuclear Phagocytes* (eds O. Forster and M. Landy), p. 153. Academic Press, New York.

BULLEN J.J., ROGERS H.J. & LEIGH J. (1972) Iron-binding proteins in milk and resistance to *Escherichia coli* infections in infants. *Br. med. J.* **5**, 69.

GORDON G.W., ZIEGLER J. & BASCH J.J. (1962) Isolation of an iron-binding protein from cow's milk. *Biochem. biophys. Acta*, **60**, 410.

VAN HAERINGEN N.J. (1981) Clinical biochemistry of tear fluid. *Surv. Ophthalmol.* **26**, 84.

HEKMAN A. (1971) Association of lactoferrin with other proteins, as demonstrated by changes in electrophoretic mobility. *Biochem. biophys. Acta*, **251**, 380.

KIJLSTRA A., JEURISSEN S.H.M. & KONING K.M. (1984) Complement inhibitors in tears of different species. *Exp. Eye Res.* **38**, 57.

KIJLSTRA A. & JEURISSEN S.H.M. (1982) Modulation of classical C3 convertase of complement by tear lactoferrin. *Immunology*, **47**, 263.

KIJLSTRA A., JEURISSEN S.H.M. & KONING K.M. (1983) Lactoferrin levels in normal human tears. *Br. J. Ophthalmol.* **67**, 199.

KIJLSTRA A. & VEERHUIS R. (1981) The effect of an anticomplementary factor on normal human tears. *Am. J. Ophthalmol.* **92**, 24.

MASSON P.L. & HEREMANS J.F. (1968) Metal-combining properties of human lactoferrin. *Eur. J. Biochem.* **6,** 578.

MASSON P.L., HEREMANS J.F. & DIVE C. (1966) An iron-binding protein common to many secretions. *Clin. Chim. Acta,* **14,** 753.

MASSON P.L., HEREMANS J.F. & SCHONNE E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leucocytes. *J. exp. Med.* **130,** 643.

MAZURIER J., AUBERT J.P., LOUCHEUX-LEFEVRE M.H. & SPIK G. (1976) Comparative circular dichroism studies of iron free and iron saturated forms of human serotransferrin and lactotransferrin. *FEBS Lett.* **66,** 238.

MONDINO B.J. & ZAIDMAN G.W. (1983) Hemolytic complement in tears. *Ophthalmic Res.* **15,** 208.

VAN SNICK, J.L. & MASSON P.L. (1976) The binding of human lactoferrin to mouse peritoneal cells. *J. exp. Med.* **144,** 1568.

VEERHUIS R. & KUILSTRA A. (1982) Inhibition of hemolytic complement activity by lactoferrin in tears. *Exp. Eye Res.* **34,** 257.